Dysfunctional Vasa Vasorum in Diabetic Peripheral Artery Obstructive Disease with Critical Lower Limb Ischaemia


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Submitted 27 November 2009; accepted 26 April 2010
Available online 1 June 2010

KEYWORDS
Diabetes; Peripheral arterial obstructive disease; Vasa vasorum; Resident endothelial progenitor cells; Angiogenesis

Abstract
Objectives and design: To establish whether in diabetic patients with peripheral artery obstructive disease (PAOD) vasa vasorum (vv) neoangiogenesis is altered with increased arterial damage.

Materials: Thirty-three patients with PAOD and critical lower limb ischaemia, 22 with type II diabetes.

Methods: Immunohistochemistry for endothelial cell markers (CD34 and von Willebrand Factor); real-time reverse transcription polymerase chain reaction (RT-PCR) to quantify arterial wall expression of vascular endothelial growth factor (VEGF); enzyme-linked immunosorbent assay (ELISA) to assess blood VEGF; flow cytometry to detect circulating endothelial cells (CECs).

Results: Patients with PAOD and diabetes have a higher frequency (60% vs. 45%) of advanced atherosclerotic lesions and a significant reduction ($p = 0.0003$) in CD34$^+$ capillaries in the arterial media. Adventitial neoangiogenesis was increased equally (CD34$^+$ and vWF$^+$) in all patients. Likewise, all patients have increased CEC and VEGF concentration in the blood as well as in-situ VEGF transcript expression.

Conclusions: Patients with PAOD have remarkable arterial damage despite increased in-situ and circulating expression of the pro-angiogenic VEGF; a dysfunctional vv angiogenesis was...
One of the most dramatic consequences of peripheral arterial obstructive disease (PAOD) is the critical lower limb ischaemia (CLI), a clinical condition often followed by the onset of gangrene and subsequent major amputation. This condition is greatly enhanced by the presence of diabetes.  

Diabetic arteriopathy is characterised by arterial narrowing due to the rapid progression of atherosclerosis; blood endothelial progenitor cells (EPCs) numeric depletion is also observed as well as their functional impairment with reduced adhesion, migration and incorporation into tubular structures. Moreover, an increased presence of mature circulating endothelial cells (CECs) has been reported in conditions associated with arterial injury; these cells representing the straight evidence of endothelial damage in the intima.  

In PAOD, neoangiogenesis plays a key role when exposed to hypoxia, and resident vascular cells are activated and release growth factors. The vascular endothelial growth factor (VEGF) is essential in the regulation of neoangiogenesis; through binding to its cognate receptor, VEGF promotes a multistep process which ends with endothelial cell (EC) differentiation into mature blood vessels. VEGF also contributes to target EPCs in the injured sites where they synergistically co-operate with resident cells in promoting effective angiogenesis and arteriogenesis. Recently, a subset of EPCs has been found within the vascular wall; these resident EPCs are located in correspondence with the vasa vasorum plexus in the human arterial wall; vasa vasorum are supposed to contribute to vascular wall homeostasis during adult life and are known to respond dynamically to risk factors for atherosclerosis, such as hypercholesterolaemia and hypertension; the role of vasa vasorum in diabetic arteriopathy remains quite unexplored.  

In this study we confirm our hypothesis that, in diabetic patients with PAOD and CLI, vasa vasorum are unable to carry on an effective arterial wall neoangiogenesis; in-situ altered angiogenesis may contribute to accelerated parietal damage commonly seen in diabetic patients. For this purpose, we studied ‘in-situ’ expression of the two endothelial markers, CD34 and von Willebrand Factor (vWF); the severity of parietal damage, by means of histopathological evaluation and flow cytometry assessment of CEC number; in-situ and circulating expression of the pro-angiogenic VEGF.

Materials and methods

Patients

Thirty-three patients with PAOD and CLI were recruited from the Vascular Surgery Unit of the S. Orsola-Malpighi Hospital of Bologna, after obtaining the approval of the ethics committee. For flow cytometry, serological and molecular studies, healthy subjects (n = 29) and multi-organ donors (n = 3) were selected as controls, respectively, from the Transfusion Centre and the Cardiovascular Tissues Bank of the S. Orsola-Malpighi Hospital. For histopathological evaluation and immunohistochemical analysis control, femoral arteries (n = 10) were recovered from the Surgical Pathology’s archive, Department of Haematology, Oncology and Laboratory Medicine, S. Orsola-Malpighi Hospital.  

The diagnosis of peripheral arteriopathy was made considering not only the vascular health conditions but also risk factors such as smoke, diabetes mellitus, hypertension, hyperlipidaemia and related pathologies such as chronic renal insufficiency (IRC), ischaemic heart disease, cerebrovascular insufficiency (ICV) and chronic obstructive pulmonary disease (COPD). PAOD clinical stage was established according to Fontaine-Leriche and Texas University classifications; arterial lesion topography was evaluated by means of Doppler ultrasonography, with or without angiography. Once the severity of the disease and the concrete risk of lower limb amputation were confirmed, patients underwent surgical revascularisation. During surgery, arterial wall fragments were taken from the proximal anastomosis of the bypass, and destined to histopathological, immunohistochemical and molecular analyses. Before the surgical treatment, peripheral blood samples of 40 ml were taken from each patient and used for flow cytometry and enzyme-linked immunosorbent assay (ELISA). Our experimental strategy is reported in Diagram 1.

Arterial wall histopathological evaluation

Arterial tissue samples were fixed in formalin and embedded in paraffin; 5-μm-thick haematoxylin- and eosin (H&E)-stained sections were observed under a light microscope (LM, Olympus CX42), using healthy femoral arteries recovered from our archive as controls.  

Arterial wall lesions were classified according to the American Heart Association (AHA) classification and grouped as class I (arterial wall samples with type I–III lesions) and class II (arterial wall samples with type IV–VI lesions).

Immunohistochemical assay of arterial wall neoangiogenesis

Angiogenesis was investigated by immunohistochemical detection of CD34, a marker of EPCs and microvascular endothelial cells (ECs), and von Willebrand Factor (vWF), a protein expressed by mature endothelium. Formalin-fixed paraffin-embedded 3-μm-thick tissue sections were dehydrated and rehydrated through decreasing concentrations of ethanol. Tissue antigenicity was recovered at 1 atm, 120°C for 20 min. Endogenous peroxidase activity was blocked with 3% H2O2 in absolute methanol for 10 min at room temperature (rt). Antigen–antibody reaction was developed with the NovoLink Polymer Detection Kit (Novocastra,  

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Sections were incubated overnight in a wet chamber at 4°C with monoclonal antibodies against CD34 (clone QBEnd-10; 1:80; Dako, Copenhagen, Denmark) and vWF (clone F8/86; 1:50; Dako, Copenhagen, Denmark); then, sections were incubated with NovoLink™ Polymer for 30 min at rt, and subsequently with diaminobenzidine (NovoLink™ DAB Substrate Buffer) for 30 s. Cell nuclei were stained with Mayer’s haematoxylin (Sigma Chemicals). Negative controls were done by omitting incubation with the primary antibody. The samples were observed in a computer-assisted light microscope using Image-ProPlus software (ver. 4.5; MediaCybernetics http://www.mediacy.com). The evaluation was performed on at least 10 random fields taken at 20× magnification in each arterial section; arterial media and adventitia layers were recorded separately. Healthy femoral arteries, recovered from our files, were used as positive controls. Capillary density was evaluated as previously described. In-situ VEGF mRNA expression

VEGF mRNA transcripts were quantified in arterial tissue samples by real-time reverse transcription polymerase chain reaction (RT-PCR) analysis as previously reported; fresh artery samples recovered from healthy multi-organ donors were considered controls. The samples were stored at −80°C in RNA-free eppendorf tubes containing a lysis and nucleic acids purification solution (Lysis Solution 2X), homogenised through centrifugation and, then, digested with proteinase K (200 µg ml⁻¹ of Lysis Solution) overnight at rt. The samples were centrifuged at 14,000 rpm for 5 min and the aqueous phase containing RNA was recovered. Total RNA extraction was performed with an ABI PRISM 6100 Nucleic Acid Pre-station (Applied Biosystems, USA) and its related chemistry. RNA was dried using a UNI100H drier (UNIEQUIP). Subsequently, the RNA was dissolved in RNase-free water (DEPC water) and quantified with a Nanodrop spectrophotometer (ND-420, Nanodrop Technology). Nucleic acid quality was assessed measuring the A260/A280 ratio. Ten microlitres of total RNA were used for RT reaction following the manufacturer’s protocol (High capacity cDNA Archive kit, Applied Biosystems, USA). cDNA was then amplified through a real-time PCR. The reaction mix (all reagents from Applied Biosystems) contained TaqMan DNA polymerase (TaqMan® Universal PCR Master Mix 2X) and primers and probes for VEGF (TaqMan® Gene Expression Assays – Hs00900054_m1- 20X); as housekeeping gene β-actin (TaqMan® Gene Expression Assays – Hs 99999903_m1- 20X-FAM) was used. Real-time PCR was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA).

ELISA assay

Serum VEGF concentration was determined with an ELISA using the Quantikine kit (R&D Systems, Minneapolis, MN, USA). Donor serum samples were controls. According to the

Diagram I  Layout of the experimental strategy.
manufacturer’s instructions, samples were pipetted in a 96-well polystyrene microplate pre-coated with a monoclonal antibody specific for VEGF; after washing, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. After additional washings, a substrate solution was layered on each well and a colorimetric reaction developed. The intensity of colour, which was proportional to the amount of VEGF bound in the initial step, was measured using a multiplate spectrophotometer reader at an optical density of 450 nm.

Detection of CD146⁺ circulating endothelial cells

Flow cytometry was performed on peripheral blood samples for determining CEC levels. The assay was performed with the CELLQUANT FF-CD146 Kit (BioCytex, France); healthy subject blood samples were used as control after informed consent was obtained. Briefly, CECs were enriched from whole blood by magnetic nanoparticles coated with CD146. The enriched samples were stained with FITC-CD45 and PE-CD146 following the manufacturer’s instructions. CECs were counted using fluorescent counting beads provided with the kit. Analysis was performed with an FC500 cytometer (Beckman Coulter Miami, FL, USA). CECs were defined as cells with a CD146⁺/CD45⁻ phenotype per millilitre.

Statistical analysis

Data have been summarised as appropriate means and standard deviations, but generally medians and interquartile ranges (IQRs) were preferred owing to the non-normal distribution of measures. Consequently, for comparisons, non-parametric tests were performed (Mann–Whitney U test), and scatter graphs were used for graphic representations.

Data in nominal or ordinal scale were summarised as numbers of subjects and percentages, and Fisher’s exact test or raw OR, ORMH were used for comparisons.

Due to the relatively small number of subjects and of controls, it was not possible to correct some comparisons for age and gender; when possible, data were put into a backward-logistic regression model to calculate a corrected odds ratio (OR) between cases and controls. Statistical analysis was carried out using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). A first type error <0.05 was accepted.

Results

Patients and controls

Patients’ age ranged from 39 to 91 years with a mean value of 71.3 ± 10.8.

Twenty-two PAOD patients (66.7%), suffering from type II diabetes, constituted the diabetic PAOD group, and 11 patients (33.3%) were available in the non-diabetic PAOD control group. Although diabetic subjects were under hypoglycaemic therapy (85% treated with insulin, 15% with oral hypoglycaemic agents), they had a mean glycaemic value (159 ± 76 mg dl⁻¹) out of the normality range (60–110 mg dl⁻¹). Patients were distinguished in 24 males (72.7%) and nine females (27.3%), with males being significantly older than females (p = 0.044). No statistically significant difference was revealed for diabetes frequency in the two genders.

From healthy controls, none of them were suffering from diabetes. No significant difference was shown between patients and controls for gender or age.

The prevalence of risk factors and the severity of the disease for the patients are summarised in Table 1.

Morophological analysis

Diabetic arterial wall compared with non-diabetic one revealed a higher frequency (60% vs. 45%) of advanced lesions; such difference was not statistically significant, particularly when corrected for age and gender. No significant difference was found between the two groups of patients (Fig. 1A–D).

Arterial wall neoangiogenesis

Vasa vasorum in the adventitia were lined with vWF⁺/CD34⁻ ECs; on the contrary, capillaries in the arterial media predominantly expressed the CD34 surface molecule. When
compared to healthy controls, quantitative evaluation of immunohistochemical staining in PAOD patients revealed a significant increase in adventitial neoangiogenesis, CD34\(^+\) and vWF\(^+\) (\(p = 0.006\) and \(p = 0.0001\), respectively); no significant difference was seen between CD34 and vWF expression in PAOD patient adventitia layer (Fig. 2A–G). In contrast, in PAOD patient media layer CD34\(^+\) neoangiogenesis was significantly higher than vWF\(^+\) angiogenesis (\(p = 0.0001\)) (Fig. 3A–E); however, the diabetic group showed a significantly reduced CD34\(^+\) medial neoangiogenesis (\(p = 0.0003\)) when compared to the non-diabetic control group (Fig. 4A–D).

**Arterial wall expression of VEGF mRNA transcripts**

Real-time RT-PCR revealed a significantly higher expression of VEGF mRNA transcripts in PAOD patients than in healthy controls (\(p = 0.016\)) (Fig. 5A).

The comparison patient-control age-twined confirmed this significant difference (\(p = 0.034\)), indicating that the increase of in-situ VEGF expression was related to the peripheral arteriopathy, but was not influenced by patients’ age.

No significant difference was revealed between diabetic and non-diabetic patients.

**Blood VEGF**

Serum VEGF concentration resulted in a significant increase in all PAOD patients (median 170,775 and IQR from 45,91 to 330,778) compared to healthy controls (median 75,775 and IQR from 12,168 to 147,723) (\(p = 0.036\)) (Fig. 5B). Such difference was not statistically significant after comparison of patient-control age-twined, suggesting an influence of patients’ age on circulating VEGF level increase in PAOD subjects with respect to controls. No difference was revealed between diabetic and non-diabetic patients.

**Flow cytometry of circulating endothelial cells**

Median values of CECs per millilitre resulted significantly higher in all PAOD patients (369,5 and IQR from 221,5 to 835) than in control healthy subjects (110 and IQR from 75,5 to 238,5) (\(p = 0.0001\)) (Fig. 5C), with diabetic subjects presenting by far the highest values. A backward-logistic regression, performed to correct data for age and gender, confirmed this significant difference (\(p = 0.03\)), indicating that the subjects with peripheral arteriopathy, with respect to healthy subjects, had an increased CEC number in the peripheral blood, independent of their age and sex. No significant difference was seen between the two PAOD populations.

**Discussion**

Diabetes mellitus represents one of the greatest medical and socio-economic challenges worldwide\(^\text{17}\) with the incidence of type II disease already reaching epidemic...
Figure 2  Quantitative immunohistochemistry shows a significant increase in adventitial neoangiogenesis (CD34⁺ and vWF⁺) in all PAOD patients (diabetic and non-diabetic). A), C) and E) immunohistochemical CD34 detection; B), D) and F) immunohistochemical vWF analysis; G) PAOD patients, when compared to healthy controls, present a significant increase in CD34 and vWF expression in adventitia layer (p = 0.006 and p = 0.0001, respectively); on Y axis, the values are referred to positive areas (μm²) observed for the two endothelial markers. Ctr: control femoral artery from healthy multi-organ donor; nd: tissues from non-diabetic patients; d: tissues from diabetic patients. 100x, original magnification (250 μm, scale bar) for A) and B); 25x, original magnification (100 μm, scale bar) for C) and E); 25x, original magnification (250 μm, scale bar) for D) and F).
proportions. Despite the introduction of new therapies, vascular complications represent the leading cause of morbidity and mortality in diabetic patients. People with diabetes have a much higher incidence of coronary artery disease as well as peripheral artery disease, and cerebro-vascular disease rather than the general population in part due to accelerated atherogenesis. Diabetic vasculopathy is characterised by structural changes of large and small arteries with tissue hypoperfusion and hypoxia, involving multiple distal arterial segments. At the macro-vascular level, these changes manifest with the characteristic intima and media—thickening, and vessel rigidity reflecting the altered composition and function of the arterial wall. The most described features include EC dysfunction and depletion; smooth muscle cell proliferation; macrophage infiltration; changes in the extracellular matrix (ECM) composition; calcium and matrix protein deposition; and changes in the interaction between circulating cells and ECs.

Vasa vasorum are considered to play a role in the initiation, progression and complication of atherosclerosis. In large and medium-sized vessels, they provide nutrients and oxygen to the media layer while removing ‘waste’ catabolic products. On the other hand, they are thought to facilitate the entry of pro-inflammatory and pro-atherosclerotic components into the arterial wall. Further, vasa vasorum possibly represent the stem cell niche which regulates vessel wall homeostasis during the adult life. However, the role of vasa vasorum in diabetic peripheral arteriopathy yet remains quite unexplored.

In this study, a significant damage of the arterial wall with vascular endothelium injury has been documented in PAOD patients; CEC quantitative flow cytometry assay demonstrated that CD146 positive circulating ECs are significantly

![Image of Figure 3 Medial neoangiogenesis: a comparison between CD34 and vWF immunohistochemical expression in all PAOD patients. A) and C) CD34 detection; B) and D) vWF staining; E) in PAOD patient media layer, CD34+ neoangiogenesis was significantly higher than vWF+ angiogenesis (p = 0.0001); on Y axis, the values are referred to positive areas (µm²) observed for the two endothelial markers. nd: tissues from non-diabetic patients; d: tissues from diabetic patients. 10x, original magnification (100 µm, scale bar) for A), B), C) and D).]
raised in the peripheral blood of all PAOD patients, in an independent way from patients’ age, and with diabetic subjects presenting by far the highest values. The flow cytometry result is consistent with histopathological investigation that revealed a higher frequency of advanced lesions in the arterial wall of diabetics. An increase in CEC number has been described in different pathological settings characterised by vascular damage.6,7 The prolonged endothelial insult is believed to be responsible for sloughing off the vessel wall that releases in the peripheral blood circulating ECs; as previously reported,7 CEC appearance in the blood is the consequence of a disease process that irreversibly damages the endothelium. Overall, the flow cytometry and histopathological results confirm that the presence of diabetes is associated with an incremental damage of the arterial wall.

Metabolic, humoral and haemodynamic factors, all contribute to vascular injury in diabetes through growth factor and cytokine arterial wall modulation; among the growth factors, VEGF has attracted much attention because of its key role in the pathological neovascularisation that characterises diabetic microangiopathy; VEGF is a major mediator of neoangiogenesis in physiological and

Figure 4  A), B) and C) CD34⁺ capillaries in the media layer of PAOD patients (10x, original magnification; 100 μm, scale bar); D) diabetic subjects, when compared to non diabetics, showed a significant reduction in CD34⁺ medial neoangiogenesis (p = 0.0003); on Y axis, the values are referred to areas (μm²) positive for CD34. nd: tissues from non-diabetic patients; d: tissues from diabetic patients.
pathological conditions with crucial roles in developmental blood vessel formation and regulation of hypoxia-induced tissue angiogenesis. In diabetes, there is an impairment of the endogenous reperfusion mechanism with limited generation of arterial collaterals and new vessels by angiogenesis and angiogenesis; thus, a significant modulation of VEGF is expected in the context of PAOD with CLI.

In response to tissue ischaemia, the target organs increase VEGF transcript and protein levels. Accordingly, in this study, ELISA assay revealed a significant increase of serum VEGF concentration in all patients with CLI; this increase could be influenced by patients’ age and therefore be an expression of a generalised vascular damage as expected in our patient population. A differential regulation of VEGF and its receptors has been observed between highly vascularised tissues, such as the retina, and cardiac tissues; in particular, an increased VEGF mRNA expression was seen in the retina from diabetic rats, and related to the concomitant capillary leakage and excessive neo-vascularisation, while a decreased expression was found in ventricles from diabetic patients, and related to inadequate collateral formation in the diabetic myocardium. However, the VEGF role in other vascular sites in diabetes is not well characterised; low levels of constitutive VEGF mRNA in medium to large arteries have been observed in vivo in humans, and this expression was found restricted to vascular smooth muscle cells; because of VEGF role in promoting ‘in-situ’ angiogenesis, various stimuli relevant to the diabetic context have been reported to increase the vascular expression of VEGF including hypoxia, Advanced Glycation End-products (AGE’s) and elevated glucose concentrations. Here, we found that, when compared to controls, the femoral arterial wall of PAOD patients contains a significant raised expression of VEGF transcripts; this increase did not appear to be related to patients’ age, rather influenced by peripheral arteriopathy.

VEGF induces neangiogenesis by acting on resident vascular cells as well as by promoting the release of bone-marrow-derived EPCs in the circulation; EPCs are believed to localise specifically at sites of ischaemia where they contribute to blood vessel growth. In the presence of diabetes, EPCs are functionally impaired and show defective migration in response to VEGF and hypoxia-regulated factors, such as the stromal-derived factor, on which these cells depend for their recruitment to ischaemic sites. Further, a significant decrease in the number of EPC has also been described in patients with diabetes and PAOD, especially in the presence of ischaemic foot lesions. In the present study, we found a significant increase in arterial wall neangiogenesis in all PAOD patients, and this increase was unrelated to the presence of diabetes; therefore, we believe that in the present clinical context, the contribution of bone-marrow EPCs to arterial wall angiogenesis is of limited importance.

Immunohistochemical analysis showed a dense network of thin-walled capillaries, which intensely expressed vWF; the vascular network extended from the outer media throughout the adventitia layer; this anatomical location and morphology strongly suggest a neangiogenic response of the vasa vasorum system. In healthy humans, only vessels more than 0.5 mm lumen diameter do have vasa vasorum, which contribute to deliver nutrients and oxygen to the media; moreover, they respond dynamically to the vessel requirements by undergoing vasodilatation and vasoconstriction as well as increase in number. An intriguing finding is that EPCs were also discovered in the adult vascular wall in humans, indicating that the arterial wall is not only a destination but also a source of cells that have regenerative potential. Ingram et al. reported the existence of vessel-wall-derived EPCs without describing an exact parietal localisation of these cells. A ‘vasculogenic zone’, where EPCs reside, was identified in the wall of large and mid-sized blood vessels; this zone, located at the border between the medial and the adventitial layers, is believed to act as a source of progenitors for postnatal vasculogenesis, and anatomically corresponds to the vasa vasorum location.

Immunohistochemical analysis did not reveal a significant difference between CD34 and vWF adventitial neangiogenesis: the expression of these endothelial markers was quite similar, showing a significant increase in all PAOD patients with respect to healthy controls. Conversely, the media expression of CD34 capillaries resulted significantly higher than vWF one, in all PAOD patients compared to controls; however, diabetics, when compared to non-diabetic group, showed a significantly decreased CD34 medial angiogenesis, thus suggesting a dysfunctional angiogenesis in such patients. CD34 is a member of single-pass transmembrane sialomucin proteins, which is specifically expressed by EPCs, ECs and haematopoietic stem cells; the observation that neo-vessels in the media predominantly expressed CD34 is an evidence of their immature condition.

These results suggest that in diabetic milieu, vasa vasorum are unable to perform an effective VEGF-driven angiogenesis; the possibility that this failure may be
a consequence of resident EPC injury needs further investigation; here, a decreased neoangiogenesis in the media of diabetic PAOD patients was found; it is speculated that the disturbed angiogenesis could determine a reduced perfusion of the arterial wall, resulting in increased arterial wall damage that can contribute to the accelerated atherosclerosis in diabetes.

Funding

This work was supported by a grant from Ministero dell’Istruzione dell’Università e della Ricerca (http://www.miur.it) 2005 (diabetes and lower limb critical ischaemia; study on arterial wall damage and the role of endothelial progenitor cells in artery repair and neoangiogenesis).

Conflict of interest

None.

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